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Not all saponins have a greater antiprotozoal activity than their related sapogenins

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One sentence summary: The antiprotozoal activity is not an inherent feature of all saponins and small variations in their structure can have a significant influence on their biological activity.

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ABSTRACT

The antiprotozoal effect of saponins varies according to both the structure of the sapogenin and the composition and linkage of the sugar moieties to the sapogenin. The effect of saponins on protozoa has been considered to be transient as it was thought that when saponins were deglycosilated to sapogenins in the rumen they became inactive; however, no studies have yet evaluated the antiprotozoal effect of sapogenins compared to their related saponins. The aims of this study were to evaluate the antiprotozoal effect of eighteen commercially available triterpenoid and steroid saponins and sapogenins *in vitro*, to investigate the effect of variations in the sugar moiety of related saponins and to compare different sapogenins bearing identical sugar moieties. Our results show that antiprotozoal activity is not an inherent feature of all saponins and that small variations in the structure of a compound can have a significant influence on their biological activity. Some sapogenins (20(S)-protopanaxatriol, asiatic acid and madecassic acid) inhibited protozoa activity to a greater extent than their corresponding saponins (Re and Rh_1 and asiaticoside and madecassoside), thus the original hypothesis that the transient nature of the antiprotozoal action of saponins is due to the deglycosilation of saponins needs to be revisited.

Keywords: antiprotozoal activity; chemical structure; sapogenins; saponins

INTRODUCTION

Since the ban of antibiotic growth promoters for animal feeding in Europe, plant extracts and plant secondary metabolites have been widely investigated as biological materials to modify fermentation in the rumen (Hart *et al.* 2008). Although tannins and essential oils have been reported to have potential as antiprotozoal agents (Patra and Saxena 2011; Patra and Yu 2012), saponins have shown a more consistent inhibitory effect on rumen protozoa (Newbold *et al.* 2015). Since rumen protozoa are key in the turnover of bacterial protein in the rumen (Wallace and McPherson 1987), their elimination could increase microbial

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protein supply to the host (Newbold *et al.* 2015). Also, as protozoa harbour an active population of methanogenic archaea on their internal and external surfaces, defaunation could decrease methane production (Newbold *et al.* 2015).

Saponins consist of an aglycone or sapogenin linked to one or more sugar moieties through a glycosidic bond (Francis *et al.* 2002); the sugar fraction is often composed of monosaccharides such as D-glucose, L-rhamnose, D-galactose, D-glucuronic acid, L-arabinose, D-xylose or D-fucose (Podolak, Galanty and Sobolewska 2010). According to their sapogenin structure, saponins can be broadly classified as either triterpenoid or steroid (Wina, Muetzel and Becker 2005). The variations in the structure of saponins as well as in their bioactivity are determined by both the sapogenin and the presence of different substituents such as hydroxyl, hydroxymethyl, carboxyl and acyl groups, and the composition, linkage and number of sugar chains (Patra and Saxena 2009; Podolak, Galanty and Sobolewska 2010). Saponins are believed to form irreversible complexes with cell membrane cholesterol causing protozoa to rupture and lysis (Francis *et al.* 2002; Wina, Muetzel and Becker 2005). The antiprotozoal activity of saponins in the rumen was reported to be transitory and it has been suggested that the deglycosilation of saponins to sapogenins in the rumen decreases the antiprotozoal activity of saponins (Newbold *et al.* 1997; Teferedegne (2000). Although sapogenins have been considered to be inactive against protozoa (Wallace *et al.* 2002), no studies have yet evaluated their antiprotozoal effect in the rumen. This study was aimed at evaluating the antiprotozoal effect of saponins and their related sapogenins *in vitro*, investigating the effect of variations in the sugar moiety of related saponins, and comparing different sapogenins bearing identical sugar moieties. Furthermore, the antiprotozoal effect of a range of sapogenins is reported for the first time.

MATERIAL AND METHODS

Saponins and sapogenins

Eighteen commercially available triterpenoid and steroid saponins and sapogenins were selected based on: 1) differences in aglycone: triterpenoids of the dammarane (ginsenosides, protopanaxadiol and protopanaxatriol), ursane (asiaticoside, madecassoside and their corresponding sapogenins) and oleanane (saikosaponins) type and steroids of the spirostan type (dioscin and diosgenin) 2) differences in the sugar moieties and linkage to the sapogenin. Structure of saponins and sapogenins are shown in Figs. 1–3.

Triterpene saponins and sapogenins from *Panax ginseng* were purchased from Extrasynthese (Genay Cedex, France): 20(S)- protopanaxadiol (PPD) and the PPD-type ginsenosides Rb_1, Rb_2, Rc, Rd and Rh_2 and 20(S)-protopanaxatriol (PPT) and the PPT-type ginsenosides Re and Rh_1. Triterpene saponins and sapogenins from *Centella asiatica* (asiaticoside, madecassoside and asiatic and madecassic acids) and triterpene saponins derived from *Bupleurum falcatum L* (saikosaponins a, c and d), were obtained from ChromaDex Inc. (Irvine, California, EEUU). The steroidal saponin dioscin, which occurs abundantly in *Dioscorea alata*, *Smilax China* and *Trigonella foenum-graecum*, and its sapogenin diosgenin, were also purchased from ChromaDex Inc. (Irvine, California, EEUU). All saponins and sapogenins were provided as pure compounds and details related to purity are available from the suppliers.

Measurement of protozoal activity

The effect of saponins and sapogenins on protozoa was estimated based on the engulfment and digestion of [^{14}C]-labelled bacteria by protozoa in mixed rumen fluid (Wallace and McPherson 1987). Briefly, in this method a pure culture of a rumen bacterium (*Streptococcus bovis* ES1) is grown under anaerobic condition in a media containing [^{14}C] leucine (1.89 μCi/7.5 mL tube) as the primary N source (Wallace and McPherson 1987). Washed bacteria (0.2 mL) were then incubated anaerobically with 3 mL of rumen fluid diluted in simplex type salt solution (1:1; Williams and Coleman 1992) in the presence of excess ^{12}C-leucine and no additive (control) or 0.05, 0.1, 0.2 or 0.4 g/L of saponins or sapogenins for up to 5 h.

Saponins and sapogenins were solubilized in ethanol at 1% (v/v) which has been shown not to impair fermentation (Morgavi *et al.* 2004; Wallace *et al.* 2007). Ethanol at 1% was also included in the control treatment. Samples (0.2 mL) were withdrawn at time 0 and at 1 h intervals up to 5 h into tubes containing 0.05 mL of 25% trichloroacetic acid and centrifuged. Radioactivity released in the supernatant was determined by liquid-scintillation spectrometry (Hidex 300 SL, Lablogic Systems Ltd, Broomhill, UK). Bacterial breakdown was estimated from the percentage of the acid soluble radioactivity released relative to the total radioactivity in the initial bacteria inoculum (Wallace and McPherson 1987). Incubations were carried out using rumen fluid obtained from four rumen cannulated Holstein-Frisian cows (four replicates) fed ryegrass and concentrate (67:33 on a DM basis) at maintenance levels. The Animal Scientific Procedures Act 1986 was followed to carry out the animal procedures and Aberystwyth University Ethical Committee approved the experimental protocols.

For each treatment and dose, a linear regression of bacterial breakdown vs time (from 0 to 5 h) was conducted, the slope of this trend-line indicating the rate of bacterial degradation (as % h^{-1}) which was taken as a proxy of protozoal activity. ANOVA was then used to analyse protozoa activity (% inhibition with respect to the control) with treatment, dose and their interaction as fixed effects and cow as blocking term. Polynomial contrast was carried out to determine linear (L) and/or quadratic (Q) responses to the treatments. Genstat 16th Edition (VSN International, Hemel Hempstead, UK) was used.

RESULTS

For the control treatment, release of [^{14}C]- increased linearly ($R^2 > 0.99$) over the whole incubation. The inhibition of protozoa activity differed between compounds and doses for both ginsenosides of the PPD and PPT type (Table 1). Whereas PPD and ginsenoside Rh_2 did not have a dose dependent effect on protozoa activity, increasing levels of the rest of the PPD-type ginsenosides resulted in a linear and quadratic increase ($P < 0.001$) in the antiprotozoal effect;protozoa activity was inhibited by 75–88% when ginsenosides Rd, Rb_1, Rb_2 and Rc were added at 0.2 and 0.4 g/L. Ginsenoside Rc and Rd showed the strongest effect, inhibiting protozoa activity by 40 and 45%, respectively, when added at 0.1 g/L. In contrast, PPT inhibited protozoal activity in a dose dependent manner (linear increase, $P < 0.001$) and to a greater extent than PPD. The antiprotozoal effect of PPT was also greater than its corresponding saponins Re and Rh_1, at all doses tested (Table 1).

When studying the acute antiprotozoal activity of saponins and sapogenins from *Centella asiatica* (Table 2), differences between compounds and doses were also observed. Whereas

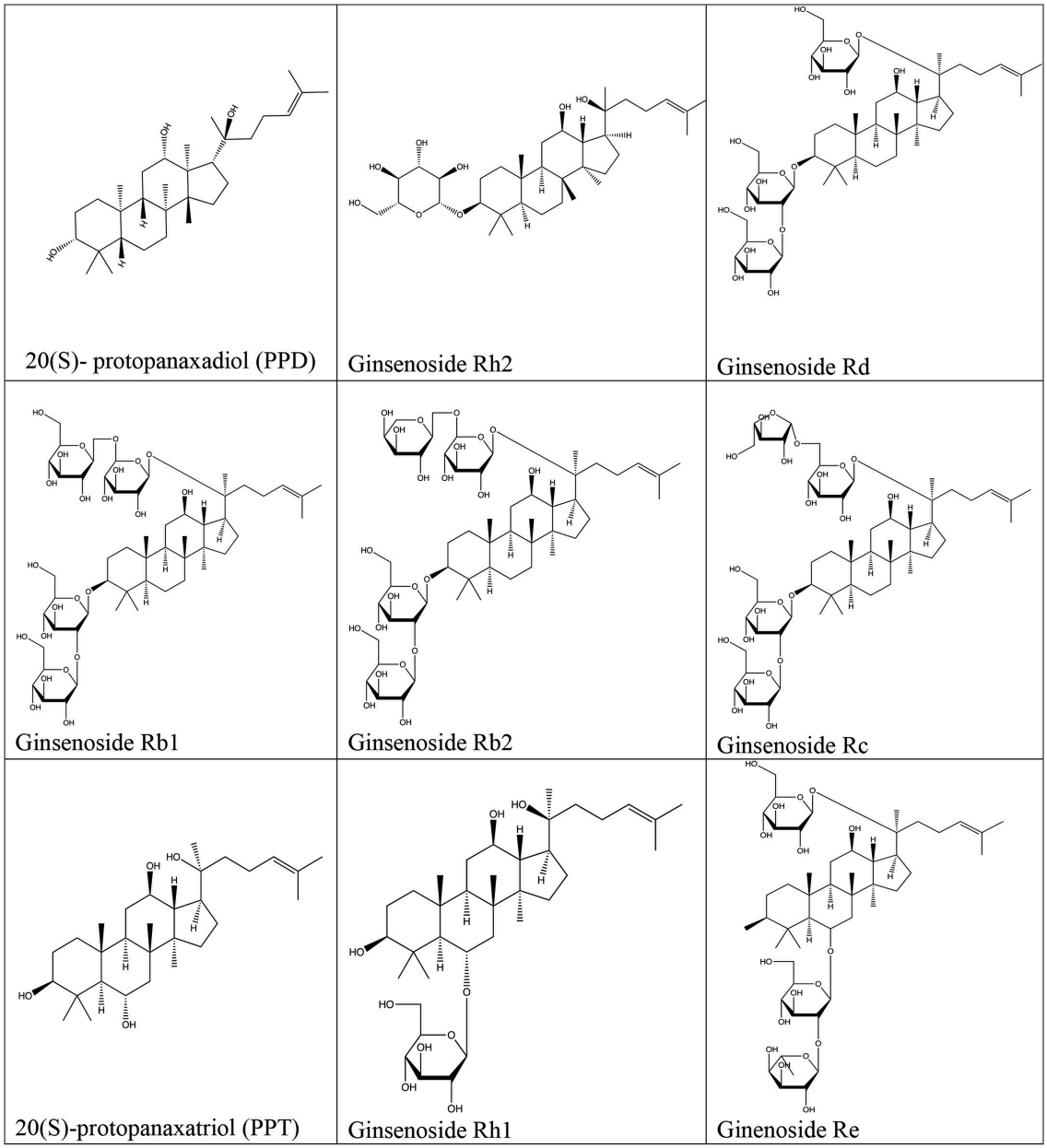

Figure 1. Structure of 20(S)- protopanaxadiol (PPD), 20(S)-protopanaxatriol (PPT), the PPD-type derivatives Rb_1, Rb_2, Rc, Rd and Rh_2, and the PPT-type derivatives Re and Rh_1.

asiaticoside and madecassoside inhibited protozoa activity by 21–32%, their corresponding sapogenins, asiatic acid and madecassic acid, caused an inhibition of 67–96%, when added at 0.2 and 0.4 g/L (Table 2); increased levels of asiatic and madecassic acids resulted in a linear increase ($P < 0.001$) in the inhibition of protozoa activity. However, the steroidal saponin dioscin was more effective at inhibiting protozoa activity than its sapogenin diosgenin with increasing levels resulting in a linear and quadratic increase ($P < 0.001$) in the antiprotozoal effects (Table 2; when added at 0.1 g/L protozoa activity was inhibited by 82% and 24% with dioscin and diosgenin, respectively). Dioscin caused almost complete inhibition of protozoa activity when added at 0.2 and 0.4 g/L (Table 2).

Differences between saikosaponins and doses tested were also observed (Table 3). Saikosaponin c inhibited protozoa activity by 22% at 0.4 g/L. Saikosaponins a and d, however, inhibited protozoa activity in a linear and quadratic manner ($P < 0.001$). An inhibition of 86 and 72% was observed when adding saikosaponins a and d at 0.4 g/L. These two saponins were also effective at 0.1 g/L (35–45% of inhibition) and 0.2 g/L (61–73% of inhibition, Table 3).

DISCUSSION

Saponins have been proposed as rumen manipulators that suppress ciliate protozoa (Wina, Muetzel and Becker 2005). The antiprotozoal effect has been associated with the sterol binding capabilities of saponins (Wina, Muetzel and Becker 2005) and protozoal species seem to have different sensitivity to saponins according to the composition of the sterols in their cellular membranes (Patra and Saxena 2009). The antiprotozoal effect of

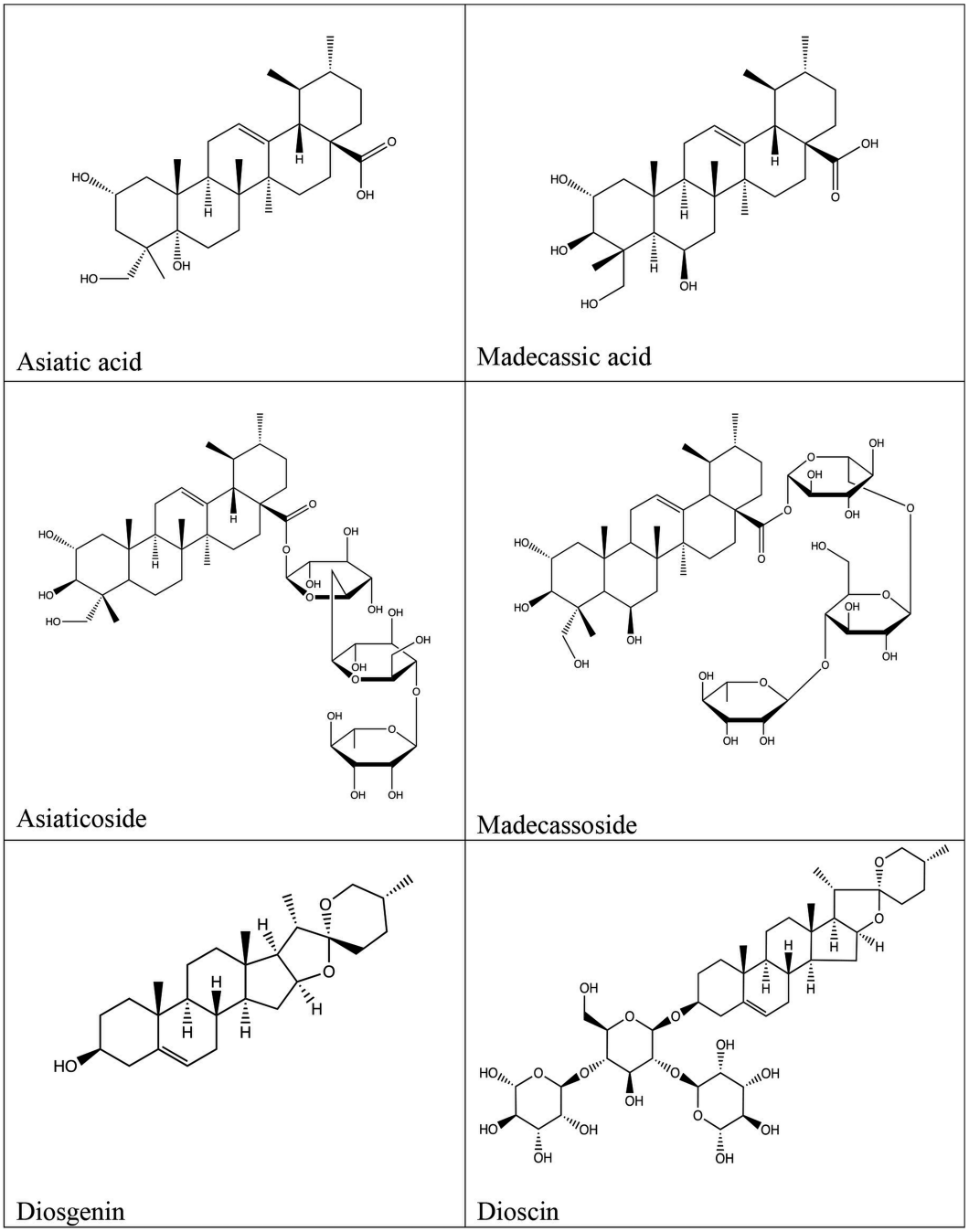

Figure 2. Structure of triterpene (asiaticoside, madecassoside) and steroidal (dioscin) saponins and their corresponding sapogenins (asiatic acid, madecassic acid and diosgenin).

saponins depends on the nature of the aglycone backbone and the number, composition and linkages of the sugar moieties (Patra and Saxena 2009). This effect is, however, lost overtime which has been attributed to the degradation of the saponins in the rumen. The only studies available on the antiprotozoal activity of saponins and sapogenins showed that regardless of the structure, saponins were more effective inhibiting protozoa than their corresponding sapogenins (Teferedegne 2000; Wallace *et al.* 2002). Thus, deglycosilation of saponins to sapogenins was suggested to explain the loss of antiprotozoal activity observed in many studies (Wallace *et al.* 2002). We have investigated this difference between saponin and sapogenin here. Thus, in our experiments we have used a short-term assay to determine protozoal activity by breakdown of ^{14}C labelled bacteria, specifically to investigate this difference between saponin and sapogenin. It should be noted that *in vivo* the antiprotozoal activity will be affected by degradation of the saponin, the mixed microbial population apparently has a very limited ability to degrade sapogenins (Wang *et al.* 1998). Thus, the results presented here do not provide information on the likely effect *in vivo*. Furthermore, we acknowledge other antimicrobial activities of saponins, including studies suggesting that sugar components are crucial for antifungal effects (Osbourn 2003) and others reporting a greater effect of sapogenins on

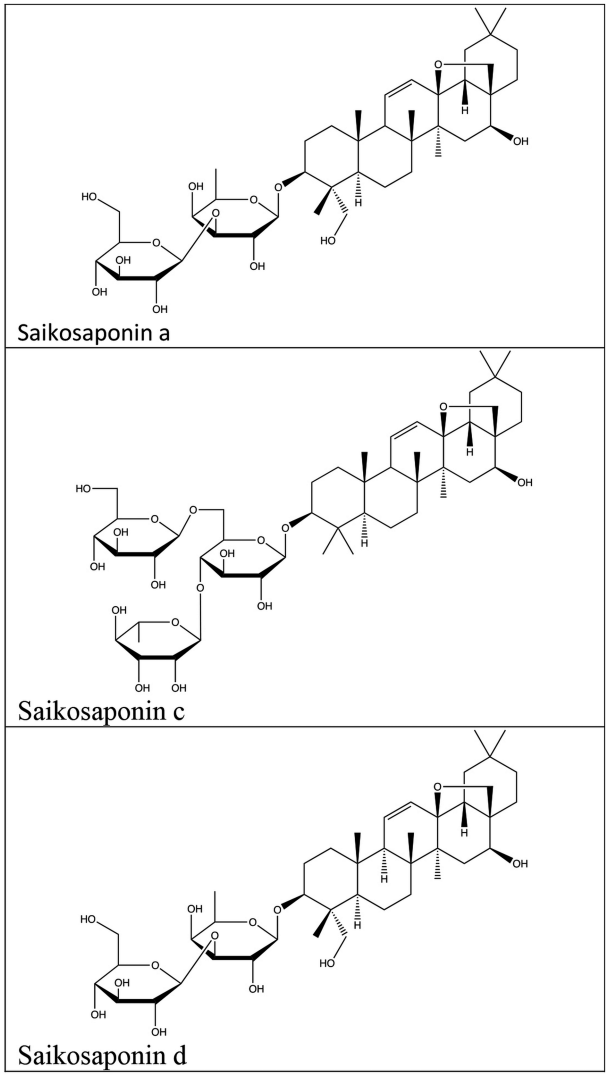

Figure 3. Structure of saikosaponins a, c and d.

yeast and bacteria compared to saponins (Avato *et al.* 2006), that imply that our studies, focussed on antiprotozoal activity, do not fully explain the differences of saponins and sapogenins on rumen fermentation.

In this work, we studied the antiprotozoal effect of a range of different saponins and sapogenins available commercially in an attempt to establish a structure activity relationship in order to ultimately identify compounds that can be used effectively as antiprotozoal agents. We also revisited the concept of the biological inactivity of sapogenins compared to saponins. Ginsenosides, triterpene saponins found nearly exclusively in Panax species, are generally composed of a dammarane skeleton (17 carbons in a four-ring structure) with different sugar moieties attached to the C-3 and C-20 positions (Leung and Wong 2010). Over 30 identified ginsenosides are classified as either PPD-type ginsenosides (Rb_1, Rb_2, Rb_3, Rc, Rd, Rg_3, Rh_2, Rs_1) or PPT-type ginsenosides (including Re, Rf, Rg_1, Rg_2, Rh_1), depending on the absence or presence of a hydroxyl group/sugar residue at C-6, respectively (Leung and Wong 2010). Our results showed that although PPD had little antiprotozoal effect, an increased inhibition of protozoal activity was observed with increasing concentrations of PPT. However, the esterification of the hydroxyl group at C-6 with glucose/rhamnose-glucose and/or the glucose attached to C-20 (resulting in the PPT-type ginsenosides Rh_1 and Re, respectively) reduced the antiprotozoal effect.

Based on the monosaccharide components in the sugar chain, ginsenosides are classified as Rx as their polarity decreases from an index of a to h (Leung and Wong 2010). If the antiprotozoal effect of saponins was exclusively related to their polarity, ginsenosides Rb and Rc should have had the greatest antiprotozoal effect; this was not so thus other factors must play a role in the antiprotozoal activity of saponins. Ginsenosides Rh_1 and Rh_2, which have same substituent but different aglycones (PPT and PPD, respectively), showed similar effects.

Interestingly, incubations with saponins and sapogenins from *Centella asiatica* showed a greater antiprotozoal effect of the sapogenins (asiatic and madecassic acids) compared to the saponins (asiaticoside and madecassoside). Indeed, the antiprotozoal effect of asiatic acid and madecassic acid increased in a dose dependent manner causing almost the total inhibition of the protozoa activity when added at 0.4 g/L. A low haemolytic activity of saponins from *Centella asiatica* as compared to their sapogenins has been also reported (James and Dubery 2009). Similarly, Vo, Fukushima and Muranaka (2017) found a haemolytic effect with asiatic acid but not with asiaticoside which was attributed to the glycosylation at C-28 reducing the haemolytic effect. The same structural effect may explain the lack of antiprotozoal activity observed with asiaticoside and madecassoside as compared to the corresponding sapogenins asiatic acid and madecassic acid. It may also be that the inhibition of protozoa activity by asiatic acid and madecassic acid was perhaps not a consequence of the permeabilization of the protozoa membrane but rather different mechanisms such as morphological and sterol level alterations, as suggested by Medina *et al* (2015) when testing tomatine, a saponin like-compound, and tomatidine, its sapogenin for their antiprotozoal effect.

The steroidal saponin dioscin showed a greater antiprotozoal effect than its sapogenin diosgenin; indeed, dioscin was remarkably active against protozoa even at low concentrations (0.1 g/L). Our results are in agreement with those of Teferedegne (2000) who also showed no antiprotozoal activity for diosgenin. These authors did not observed antiprotozoal activity when incubating digitonin, a glycoside of diosgenin, which differs from dioscin in the number and type of sugars attached to C-3. These differences in activity between digitonin and dioscin show that different numbers and sugars types attached at the same position in the aglycone confer different antiprotozoal activity to the resultant saponins.

Different antiprotozoal effects were also observed when testing the three major saikosaponins in *Bupleurum falcatum*, saikosaponin a, c and d. Saikosaponins a and d, which only differ in their aglycone configuration at C-16 (16α-OH and 16β-OH, for saikosaponins d and a, respectively, having similar substituent at C-23 (OH) and C-3 (β-D-Glu-(1→3) -β-D-Fuc)), had a similar effect and a greater effect than that of saikosaponin c (C-16: β-OH, C-23:H, C3: β-D-Glu-(1→6) –[α-L-Rha-(1→4)] β-D-Glu)). This observation agrees with a recent review on the pharmacological effects of saikosaponins (Li *et al.* 2018) that shows that saikosaponins d and a are the most active saponins of *Blupleurum*.

Our results showed that the antiprotozoal activity is not an inherent feature of all saponins, as previously suggested by Teferedegne (2000). Also, our observations suggest that small variations in the structure of a compound can have a significant influence on their biological activity. Since some sapogenins inhibited protozoa activity to a greater extent than their corresponding saponins, it can no longer be assumed that the antiprotozoal

Table 1. Inhibition of protozoa activity (% in respect to the control, no addition) by 20(S)- protopanaxadiol (PPD), 20(S)-protopanaxatriol (PPT), the PPD-type ginsenosides Rh_2, Rd, Rb_1, Rb_2 and Rc, and the PPT-type ginsenosides Re and Rh_1, added at 0.05, 0.1, 0.2 and 0.4 g/L.

	dose (g/L)				
	0.05	0.1	0.2	0.4	Contrasts
Protopanaxadiol (PPD)	16.0	13.1	20.8	18.1	–
Ginsenoside Rh_2	2.6	13.4	7.6	11.1	–
Ginsenoside Rd	12.3	44.7	88.1	86.5	L**Q**
Ginsenoside Rb_1	8.3	33.4	80.2	88.2	L**Q**
Ginsenoside Rb_2	12.3	30.9	75.0	88.4	L**Q**
Ginsenoside Rc	21.3	39.9	79.5	85.9	L**Q*
	SED		P		
Treatment	3.13		<0.001		
Dose	2.56		<0.001		
Treatment x Dose	6.26		<0.001		
Protopanaxatriol (PPT)	15.0	25.0	34.6	41.3	L**
Ginsenoside Re	6.7	0.1	0.2	7.6	–
Ginsenoside Rh_1	6.4	10.7	13.0	26.6	L**
	SED		P		
Treatment	2.39		<0.001		
Dose	2.76		<0.001		
Treatment x Dose	4.77		0.009		

L: linear response; Q: quadratic response; *:$P < 0.05$; **$P < 0.001$

Table 2. Inhibition of protozoa activity (% in respect to the control, no addition) by saponins (asiaticoside and madecassosice) and sapogenins (asiatic and madecassic acids) from *Centella asiatica* and saponins and sapogenins from *Trigonella foenum-graecum* L. (diosgenin and dioscin) added at 0.05, 0.1, 0.2 and 0.4 g/L.

	dose (g/L)				
	0.05	0.1	0.2	0.4	Contrast
Asiaticoside	14.3	17.7	21.0	25.8	–
Asiatic acid	16.6	24.5	66.5	92.6	L**
Madecassoside	15.9	17.0	31.9	29.1	–
Madecassic acid	14.3	27.9	68.7	96.3	L**
	SED		P		
Treatment	4.81		<0.001		
Dose	4.81		<0.001		
Treatment x Dose	9.62		<0.001		
Dioscin	11.2	82.1	96.5	99.0	L**Q**
Diosgenin	12.7	24.4	42.7	41.9	L*
	SED		P		
Treatment	5.36		<0.001		
Dose	7.58		<0.001		
Treatment x Dose	10.72		0.002		

L: linear response; Q: quadratic response; *:$P < 0.05$; **:$P < 0.001$

Table 3. Inhibition of protozoa activity (% in respect to the control, no addition) by saponins from *Bupleurum falcatum* (saikosaponins a, c and d) added at 0.05, 0.1, 0.2 and 0.4 g/L.

	Dose (g/L)				
	0.05	0.1	0.2	0.4	Contrasts
Saikosaponin a	13.5	35.2	73.0	86.4	L**Q**
Saikosaponin c	13.0	14.3	20.5	21.9	–
Saikosaponin d	11.6	44.9	61.4	71.7	L**Q**
	SED		P		
Treatment	3.73		<0.001		
Dose	4.31		<0.001		
Treatment x dose	7.46		<0.001		

L: linear response; Q: quadratic response; *:$P < 0.05$; **$P < 0.001$.

effect of saponins depend on the moieties. Thus, the original hypothesis that the transient nature of the antiprotozoal action of saponins is due to the deglycosilation of saponins needs to be further investigated.

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Conflicts of interests. None declared.

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